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A Selective Phosphodiesterase-4 Inhibitor Reduces Leukocyte Infiltration, Oxidative Processes, and Tissue Damage after Spinal Cord Injury

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Abstract

We tested the hypothesis that a selective phosphodiesterase type 4 inhibitor (PDE4-I; IC486051) would attenuate early inflammatory and oxidative processes following spinal cord injury (SCI) when delivered during the first 3 days after injury. Rats receiving a moderately severe thoracic-clip-compression SCI were treated with the PDE4-I (0.5, 1.0, and 3.0 mg/kg IV) in bolus doses from 2–60 h post-injury. Doses at 0.5 mg/kg and 1.0 mg/kg significantly decreased myeloperoxidase (MPO) enzymatic activity (neutrophils), expression of a neutrophil-associated protein and of ED-1 (macrophages), and estimates of lipid peroxidation in cord lesion homogenates at 24 h and 72 h post-injury by 25–40%. The 3.0 mg/kg dose had small or no effects on these measures. The PDE4-I treatment (0.5 or 1.0 mg/kg) reduced expression of the oxidative enzymes gp91^{phox}, inducible nitric oxide synthase, and cyclooxygenase-2, and diminished free radical generation by up to 40%. Treatment with 0.5 mg/kg PDE4-I improved motor function (as assessed by the Basso-Beattie-Bresnahan scale) significantly from 4–8 weeks after SCI (average difference 1.3 points). Mechanical allodynia elicited from the hindpaw decreased by up to 25%. The PDE4-I treatment also increased white matter volume near the lesion at 8 weeks after SCI. In conclusion, the PDE4-I reduced key markers of oxidative stress and leukocyte infiltration, producing cellular protection, locomotor improvements, and a reduction in neuropathic pain. Early inhibition of PDE4 is neuroprotective after SCI when given acutely and briefly at sufficient doses.

Key words: free radical; IC486051; inflammation; lipid peroxidation; phosphodiesterase type 4 inhibitor

Introduction

Strategies to improve recovery after spinal cord injury (SCI) fall under two broad categories based on the goals of therapy. Some are pro-regenerative, as they promote axon growth, sprouting, and connectivity below the lesion. Others are neuroprotective, and some of these are immunomodulatory, as they focus on altering the inflammatory response and reducing secondary injury. One known pro-regenerative strategy increases the levels of cyclic adenosine monophosphate (cAMP) by inhibition of phosphodiesterases (PDE; Nikulina et al., 2004; Pearse et al., 2004a). An ideal therapy would have both pro-regenerative and neuroprotective elements. Increasing intracellular cAMP potentially has both effects, as it can also reduce leukocyte activation (Schultz and Folkers, 1988) and chemokine production (Zidek, 1999). Herein we evaluate the effects of PDE4 inhibition on estimates of intraspinal inflammation and oxidative injury, tissue sparing, and behavioral recovery after SCI.

Inflammation after SCI leads to lesion expansion and progressive tissue damage rostral and caudal to the injury site. Recruitment and infiltration of inflammatory neutrophils and monocyte/macrophages leads to the irreversible destruction of normal spinal cord tissue, and consequently contributes to neurological deficits (Taoka et al., 1997). Activated neutrophils and monocytes/macrophages release chemokines and cytokines, and undergo oxidative burst responses releasing proteolytic enzymes, antimicrobial proteins, and reactive oxygen species into their surrounding environment that ultimately damage cell membranes, causing the death of neurons,

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glia, and vascular cells (Bao et al., 2004b; Bao and Liu, 2004; Taoka et al., 1997; Weiss, 1989).

The intensity and duration of the inflammatory response directly relates to the intracellular concentration of cAMP (Bruno et al., 2004), a second messenger that controls many cellular processes (Beavo and Brunton, 2002). Increased intracellular cAMP contributes to inhibition of proinflammatory cell functions such as chemotaxis, degranulation, superoxide anion generation, release of IL-8, and phagocytosis in neutrophils (Ottonello et al., 1995; Pearse et al., 2004a; Pryzwansky and Madden, 2003; Rossi et al., 1998). Furthermore, monocyte adhesion and migration are inhibited by high cAMP levels, as are phagocytosis and nitric oxide production in macrophages (Aronoff et al., 2005; Rossi et al., 1998; Zhu et al., 2001). Increased cAMP reduces adhesion molecule (CD11b/CD18/L-selectin) expression on leukocytes, and leukocyte adhesion to other cells, and disrupts chemokine-induced chemotaxis (Derian et al., 1995; Harvath et al., 1991).

The predominant form of PDE in leukocytes appears to belong to the rolipram-sensitive PDE4 isozyme group (Verghese et al., 1995). Indeed rolipram is a well studied PDE4 inhibitor, and has been shown to inhibit leukocyte functions, including leukotriene production by monocytes, and to have anti-inflammatory effects *in vivo* that include inhibition of neutrophil migration (Griswold et al., 1993). These findings indirectly demonstrate a role for PDE4 in several functions of monocytes and neutrophils. Furthermore, the PDE4B subtype has been identified, using immunocytochemistry, in activated microglia of the injured spinal cord (Whitaker et al., 2008). Targeted inhibition of the PDE type 4 (PDE4), is a potentially powerful tool (Houslay and Adams, 2003), as PDE4 inhibitors suppress the production of TNF-α, the generation of reactive oxides, and the migration of neutrophils (Giembycz, 2000; Torphy, 1998). Rolipram has been shown to decrease the production of TNF-α in homogenates of the injured spinal cord and in activated human mononuclear cells (Pearse et al., 2004a; Semmler et al., 1993). The anti-inflammatory actions of PDE inhibitors such as rolipram are currently being considered as therapeutics for diseases such as asthma, chronic obstructive pulmonary disease, and rheumatoid arthritis (Giembycz, 2000; Torphy, 1998). Relevant to our study, rolipram delivery in the first 72 h after SCI in rats had neuroprotective effects, sparing oligodendrocytes from death at 27 h post-injury, an effect that may have involved abrogation of local inflammation (Whitaker et al., 2008). Rolipram has been used in combination with cellular transplant therapies, yielding cellular sparing and improved motor outcomes with the combination treatment, and in some studies with 2 weeks of rolipram treatment alone (Beaumont et al., 2009; Bretzner et al., 2010; Koopmans et al., 2009; Pearse et al., 2004a). These studies suggest neuroprotective effects of the early administration of this PDE4 inhibitor. Others have demonstrated the effectiveness of rolipram to promote regeneration after spinal cord injury due to its important actions to block growth cone collapse (Nikulina et al., 2004; Pearse et al., 2004a). The work with rolipram highlights the potential for use of a PDE4 inhibitor as a neuroprotective agent and to promote regeneration after SCI.

These promising findings prompted us to undertake a study of the effects of PDE4 inhibition for 3 days after compression SCI on the intraspinal inflammatory response and oxidative injury within the first 3 days after injury, and also to examine effects of this 3-day treatment on tissue sparing and behavioral outcomes assessed from 2–8 weeks after injury. Our goal was to establish effects that could likely be attributed only to the early neuroprotective or anti-inflammatory effects of PDE4 inhibition. Because rolipram is known to have noxious side effects (Beaumont et al., 2009; Koopmans et al., 2009), and in our pilot and published studies (Pearse et al., 2004a), we have noted adverse side effects in rats during rolipram treatment, we elected to test a newly formulated PDE4 inhibitor, IC486051, developed by the former ICOS Corporation (Bothell, WA). IC486051 is a highly selective and potent inhibitor of PDE4 (PDE4-I), and it inhibits all four PDE4 isoforms (A–D), with an IC_{50} value of 0.6 nM against recombinant human PDE4 (ICOS Corp.; Nishiguchi et al., 2007; Snyder et al., 2005). It has a selectivity of $\sim$ 10,000-fold for PDE4 compared to other PDE isozymes. In our experiments, IC486051 produced no noticeable adverse side effects in the rats. A range of doses of this PDE4-I was delivered intravenously for 72 h after SCI in the rat. We demonstrated that acute administration of the PDE4-I IC486051 reduced the early influx of leukocytes into the injured spinal cord, limited free radical formation and tissue damage, and provided neurological improvement in a dose-sensitive manner.

Methods

Spinal cord injury and PDE4 inhibitor treatment

All protocols for these experiments were conducted in accordance with the policies established by the Canadian Council on Animal Care. Female Wistar rats ($n = 65$; Charles River, St. Constant, Quebec, Canada) were used in the assessment of inflammatory and oxidative processes. In our experience, female rats have significantly fewer complications with bladder function than do males, and accordingly we use females whenever possible to avoid that confounding factor. Male Wistar rats ($n = 23$) were used to study locomotor function and mechanical allodynia as we, like others, have found that they have a more consistent development of allodynia than do females (Christensen et al., 1996; Oatway et al., 2005). The males were also used to assess histological cord damage at 8 weeks after SCI. In our previous studies the inflammatory response in male rats was similar to that in females (Mabon et al., 2000; Saville et al., 2004). Details of numbers of animals per group are shown in Table 1.

All rats were medicated with diazepam (3.5 mg/kg IP; Sabex International, Boucherville, Quebec, Canada) and atropine (0.05 mg/kg SC; Sigma-Aldrich, St. Louis, MO), and then anesthetized with halothane as described previously (Weaver et al., 2001). The T4 spinal cord segment was exposed by a dorsal laminectomy and injured, without disrupting the dura, by a 60-sec, 35-g clip compression (Weaver et al., 2001). Post-injury adequate measures were taken to minimize pain and discomfort. As required by the University of Western Ontario animal care protocol, the analgesic buprenorphine (0.05 mg/kg; Schering-Plough Ltd., Welwyn Garden City, Hertfordshire, U.K.) was administered immediately after surgery and twice daily for the following 3 days. Further treatment with analgesics was never required. Post-operative care included antibiotics (Baytril 50 mg/mL SC; Bayer Inc., Toronto, Ontario, Canada), and subcutaneous hydration with 0.9% normal saline daily for 3 days. The animals were housed on extra bedding to prevent the development of pressure and

Table 1. Details of Numbers of Rats Used in Each Experiment of the Study

MPO assay		
Uninjured	7	
SCI	*24 h*	*72 h*
Control	13	14
PDE4-I 0.5 mg/kg	4	5
1.0 mg/kg	5	5
3.0 mg/kg	4	4
ED-1 Western blot		
Uninjured	6	
SCI	*24 h*	*72 h*
Control	13	14
PDE4-I 0.5 mg/kg	4	5
1.0 mg/kg	5	5
3.0 mg/kg	4	4
Neutrophil Western blot		
Uninjured	4	
SCI	*24 h*	*72 h*
Control	12	12
PDE4-I 0.5 mg/kg	4	4
1.0 mg/kg	4	4
3.0 mg/kg	4	4
Neutrophil and ED-1 staining at 72 h		
Control	2	
PDE4-I 0.5 mg/kg	2	
TBARS assay		
Uninjured	6	
SCI	*24 h*	*72 h*
Control	13	14
PDE4-I 0.5 mg/kg	4	5
1.0 mg/kg	5	5
3.0 mg/kg	4	4

HNE Western lot		
Uninjured	6	
SCI	*24 h*	*72 h*
Control	8	10
PDE4-I 0.5 mg/kg	4	5
1.0 mg/kg	4	5
DCF assay		
Uninjured	6	
SCI	*24 h*	*72 h*
Control	10	10
PDE4-I 0.5 mg/kg	5	5
1.0 mg/kg	5	5
gp91^{phox}/ iNOS Western blot		
Uninjured	6	
SCI	*24 h*	*72 h*
Control	9	10
PDE4-I 0.5 mg/kg	4	5
1.0 mg/kg	5	5
COX-2 Western blot		
Uninjured	6	
SCI	*72 h*	
Control	10	
PDE4-I 0.5 mg/kg	5	
1.0 mg/kg	5	
BBB/pain 1–8 weeks		
Control	11	
PDE4-I 0.5 mg/kg	6	
PDE4-I 1.0 mg/kg	6	
Histology at 8 weeks		
Control	5	
PDE4-I 0.5 mg/kg	5	

In the biochemical studies the uninjured group had 7 rats in total; the SCI control group had 27 rats in total (13 at 24 h and 14 at 72 h). A subset of controls was done together with each treatment dose group so that the number of controls equaled the number of PDE4-I-treated rats in each assay. PDE4-I group totals were 27: 0.5 mg/kg (4 rats at 24 h, 5 at 72 h), 1.0 mg/kg (5 rats at 24 h, 5 at 72 h), and 3.0 mg/kg (4 rats at 24 h, 5 at 72 h). Four additional rats were used for neutrophil and ED-1 staining.

The BBB and pain scoring was done in the same 23 rats. Of the 11 controls, 6 were done with the 0.5-mg/kg treatment and 6 with the 1.0-mg/kg treatment. The rats used for histological analysis at 8 weeks were among those tested for neurological outcome. Only 5 rats per group could be analyzed due to technical problems with one spinal cord in each group.

PDE4-I, phosphodiesterase type 4 inhibitor; TBARS, thiobarbituric acid reactive substances; SCI, spinal cord injury; COX-2, cyclooxygenase-2; iNOS, inducible nitric oxide synthase; DCF, 2′-7′-dichlorofluorescein; MPO, myeloperoxidase; BBB, Basso-Beattie-Bresnahan rating scale; HNE, 4-hydroxynonenal.

friction sores. Urinary bladders were manually emptied by abdominal compression twice daily until spontaneous voiding returned at 7–10 days.

The rats received post-SCI intravenous bolus injections of the PDE4 inhibitor (PDE4-I) IC486051 (0.5 mg/kg, 1.0 mg/kg, or 3.0 mg/kg IV in 30% DMSO, a gift of the ICOS Corporation) or vehicle alone as a control according to two protocols: (1) at 2 h and 12 h after SCI (the rats were terminated at 24 h) or (2) at 2, 12, 24, 36, 48, and 60 h after SCI (the rats were terminated at 72 h or 8 weeks). Due to suboptimal efficacy, the 3.0-mg/kg dose was not tested at 8 weeks after SCI. For the behavioral studies, one cohort was tested at the 0.5-mg/kg dose, and a second at a different time at the 1.0-mg/kg dose. The interval between injections was determined by the half-life of the PDE4-I in rats, which is approximately 12 h (ICOS, unpublished information). The duration of treatment was planned to protect the cord from the peak influx of neutrophils after SCI, and from the first wave of monocytes/macrophages, a strategy that we have used successfully for other treatments (Saville et al., 2004). The drug dosing (0.05–3.0 mg/kg) was established empirically in a pilot study that examined the intraspinal influx of neutrophils and monocytes. We received guidance from previous studies showing that IC486051 inhibits PDE4 activity and has functional effects in an *ex vivo* model of urinary bladder dysfunction (Snyder et al., 2005), and that a 1.0-mg/kg dose is effective *in vivo* in a rat bladder overactivity model (Nishiguchi et al., 2007). Pharmacokinetic analysis of the 1.0-mg/kg dose by Nishiguchi and associates (2007) revealed that the plasma concentrations reached by this dose in rats would be selective for PDE4. Given the selectivity of IC486501, our 3.0-mg/kg dose would also target only PDE4. We tested no doses greater than 3 mg/kg, as this dose was marginally effective. All aspects of testing and data analysis employed a blinded design. To accomplish this,

the vials containing either the PDE4-I or vehicle were numbered following a code generated by a staff member who did not work on the study. The code was revealed only after all data were analyzed. Rats treated with IC486051 or the vehicle ate and drank normally, moved in their cages typically for cord-injured rats, gained weight following normal patterns, and showed no evidence of toxic side effects of the treatment.

Assessment of intraspinal inflammatory cells

Intraspinal inflammatory cells were detected by immunohistochemical staining of neutrophils and macrophages in spinal cord, and quantified by myeloperoxidase (MPO) assays and Western blotting of neutrophil and macrophage markers in homogenates of the T2–T6 cord as described previously (Bao et al., 2004b). Briefly, for the histological study, the animals were perfused at 72 h after SCI with cold saline, followed by 4% paraformaldehyde. A 1-cm segment of cord centered at the injury zone (T4) was removed and post-fixed in the same fixative for another 24 h at 4°C, cryoprotected in increasing concentrations of sucrose (10%, 20%, and 30% in PBS), and sectioned into 25-μm longitudinal sections. The neutrophils were detected by a rabbit anti-rat neutrophil antibody (1:20,000, a gift of Dr. Daniel Anthony, Oxford University, Oxford, U.K.), and the macrophages were detected by a monoclonal antibody to ED-1 (1:2000; Serotec, Oxford, U.K.).

MPO enzymatic activity (mostly from neutrophils and weakly from macrophages), neutrophil antigen expression, and ED-1 expression (from macrophages and microglia) were measured from homogenates of the T2–T6 cord as described previously (Bao et al., 2004b). Although MPO can be found in macrophages, immunohistological studies in cord-injured rats have shown that at 24 h and 72 h after SCI, MPO immunoreactivity is found intensely in cells with morphology typical of neutrophils, and only weakly in macrophages (Fleming et al., 2008). ED-1 immunoreactivity is seen exclusively in microglia and macrophages. For Western blotting, protein derived from spinal cord homogenates was loaded onto a 7% or 10% polyacrylamide gel and separated by SDS-PAGE using a Bio-Rad Mini-Protean 3 apparatus (Bio-Rad, Hercules, CA), then transferred to polyvinylidene difluoride (PVDF) membranes (0.45-μm pore size; Millipore, Mississauga, Ontario, Canada). The membranes were first blocked with 5% nonfat powdered milk, and then incubated with a primary antibody, anti-ED-1 (1:2000; Serotec), or anti-rat neutrophil antibody (1:20,000, a gift of Dr. Anthony), followed by incubation with horseradish peroxidase (HRP)-conjugated donkey anti-mouse secondary antibody. The signal was developed using the enhanced chemiluminescence (ECL plus) detection system (Amersham, Oakville, Ontario, Canada). Band intensity was measured using Lab Works software (UVP, Upland, CA). The membrane was then incubated in stripping buffer and re-probed with anti-β-actin (1:10,000; Sigma-Aldrich) mouse monoclonal antibody. The signal was developed as described above. The densitometric values of the neutrophil antigen or ED-1 were normalized against β-actin to control for variation in protein loading.

Assessment of lipid peroxidation

Malondialdehyde (MDA) is used as a marker for lipid peroxidation, and was quantified in the homogenates of the T2–T6 spinal cord segment using a thiobarbituric acid reactive substances (TBARS) assay as described previously (Bao et al., 2004b). A standard curve was established using MDA bis (dimethyl acetal; Sigma-Aldrich), and lipid peroxidation was expressed as nanomoles of TBARS/g tissue. Lipid peroxidation was also detected by the presence of 4-hydroxynonenal (HNE)-bound proteins in Western blots, using an anti-HNE monoclonal antibody (1:5000; Alpha Diagnostic International, San Antonio, TX) and 10% polyacrylamide gels.

Assessment of free radical formation and oxidative enzymes

We used 2′-7′-dichlorofluorescin diacetate (DCFH-DA) as a probe to assess free radical production. An aliquot of the same sample (25 μL) from the homogenates of the T2–T6 spinal cord segment was incubated with 0.1 mM DCFH-DA at 37°C for 30 min. DCFH-DA is hydrolyzed to DCFH because the diacetate group is cleaved by esterases. DCFH is then oxidized by the reactive oxygen species to form the fluorescent compound, 2′-7′-dichlorofluorescein (DCF). The DCF formation was monitored at an excitation wavelength of 488 nm and an emission wavelength of 525 nm using a fluorescence spectrophotometer as described previously (Bao et al., 2005). Background fluorescence was corrected by the inclusion of parallel blanks. The formation of reactive oxygen species was quantified using a DCF standard curve, and results were expressed as nanomoles DCF/mg protein.

The levels of iNOS, cyclooxygenase-2 (COX-2), and gp91^{phox} protein expression in the T2–T6 spinal cord were quantified by Western blot analysis using either 7% or 10% polyacrylamide gels. Western blots of protein derived from spinal cord homogenates were probed at room temperature with antibodies to iNOS (1:5000; Calbiochem, San Diego, CA), COX-2 (1:2000; Cayman Chemical, Ann Arbor, MI), or gp91^{phox} (1:1000; BD Biosciences Pharmingen, Lexington, KY) overnight, followed by incubation with HRP-conjugated anti-rabbit or donkey anti-mouse secondary antibodies. Membranes were also probed with an anti-β-actin antibody to control for variations in protein loading.

Locomotor function

The rats were acclimated to the behavioral room where locomotor testing was done for 3 days prior to SCI and treatment. Locomotor recovery of the animals was assessed from 3 days to 8 weeks after SCI by two independent observers using the 21-point Basso-Beattie-Bresnahan (BBB) open field locomotor score (Basso et al., 1995). Testing was performed twice per week, and scores were averaged to generate a weekly score. Scores of left and right hindlimbs were averaged. Higher scores (14–21) on this scale require demonstration of fine details of locomotion. The severity of injury in our studies never permitted the rats to achieve locomotion scores in the range 14–21. At 1 week after injury all rats had very low scores of approximately 1, demonstrating the intended severity of injury. For the next 2 weeks the rats showed high individual animal variability in the rate of improvement, particularly in the control group. This variability abated at 4 weeks after injury, and data collection for analysis continued at this time. As described above, the observers were blinded regarding the treatment of the animals.

Mechanical allodynia

The rats were tested for the presence of mechanical allodynia on the hindpaws before, and 2–6 weeks after SCI as described elsewhere (Oatway et al., 2005). Briefly, 1 or 2 days before SCI, the rats were acclimated for 20 min in the acrylic glass testing chamber (8×3.5×3.5 inches), consisting of plastic mesh walls and an elevated mesh floor. Pre-injury responses were determined the day before SCI, and then beginning 2 weeks after SCI, the rats were tested once per week. When testing commenced, the rats were placed in this chamber and stimulated 10 times on the plantar surface of the hindpaw using a 15-mN modified Semmes-Weinstein monofilament. Each stimulus lasted 3 sec and was separated by a 5-sec interim period. After the testing of one hindpaw, a 2-min interim period lapsed before the second hindpaw was tested. The number of avoidance responses to the 10 stimuli for each hindpaw was tabulated, and the mean number of avoidances elicited from both hindpaws was calculated. Flinching, escape, paw withdrawal and/or licking, vocalization, and biting at the filament indicated that the rat perceived the stimulus as noxious. Prior to SCI, this stimulation never elicited avoidance responses. Again, the observers were blinded regarding the treatments the animals had received.

Histological assessment

At 8 weeks after SCI, the rats were transcardially perfused with 4% paraformaldehyde, and a 10-mm spinal cord portion encompassing the injury epicenter was extracted and embedded in paraffin for coronal sectioning at 10 μm on a microtome. Every fourth section was mounted onto a series of five slides. One series was used for immunohistochemical staining with the neuron-specific marker NeuN to permit stereological quantification of neuron numbers according to previously described methods (Schaal et al., 2007). After removing the paraffin from the sections, they were rehydrated through a series of xylene and ethanol exposures. Antigen retrieval with citrate buffer at 96°C was performed for 20 min, after which the slides were cooled to room temperature and rinsed in PBS. The sections were blocked with 5% heat-inactivated goat serum (HINGS) in PBS for 1 h at room temperature, and incubated overnight at 4°C with neuron-specific mouse monoclonal antibody, NeuN (1:400; Chemicon, Temecula, CA). The sections were rinsed three times with PBS, and then incubated for 2 h with a biotin-conjugated goat anti-mouse secondary antibody (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA). The sections were washed with PBS, and incubated with streptavidin HRP for 1 h. An immunoperoxidase substrate reaction was used to develop and visualize immunoreactive cells (Vector Laboratories, Inc., Burlingame, CA), and a methyl blue counter-stain was used to identify cell nuclei.

Stereological quantification of neuronal preservation

Sections immunostained for NeuN were examined under 200× magnification using an Olympus BX 51 microscope for stereological quantification of preserved neurons, using Stereoinvestigator 7 (MicroBrightField Bioscience, Williston, VT), as employed previously (Pearse et al., 2005). Final cell counts were expressed as numbers of estimated profiles per mm^3. A coefficient of error (R.L. Shaeffer) was calculated to assess the accuracy of these stereological estimates. For the optical fractionator tool, a grid size of 80 μm × 80 μm and a counting frame of 75 μm × 75 μm was used to count the cells. NeuN-immunoreactive neurons were counted within the entire gray matter (Pearse et al., 2005) from every third coronal section (600-μm intervals) of the T3–T5 spinal cord segment. This yielded analyses from 10 coronal sections per spinal cord.

Analysis of tissue integrity

Hematoxylin and eosin (H&E), and Luxol fast blue-stained sections (400-μm intervals) of the T3–5 cord were used to quantify volumes of normal-appearing gray and white matter within the spinal cord and lesion volumes using computer-assisted microscopy and image analysis software (Neurolucida 5.04.3; MicroBrightField) at a magnification of 400× as described elsewhere (Pearse et al., 2005). Accordingly 15 sections were analyzed from each spinal cord. In each section, the perimeter (contour) of the total spinal cord section, the normal-appearing gray matter, and the normal-appearing white matter were separately traced on live images. Normal-appearing gray matter was distinguished from damaged tissue by the presence of healthy neurons and normal cellular density (without the presence of numerous nuclei, which is indicative of immune cell infiltration). Normal-appearing white matter was defined as being non-fragmented, dark Luxol fast blue staining (not pale blue), and without immune cell infiltration (Pearse et al., 2004b). Each traced section was logged into the Neurolucida software by using a serial section manager, which tracked the position of each section within the 4-mm-long Z-stack to allow volume calculations with NeuroExplorer algorithms. The total spinal cord volume and the volumes of normal-appearing gray and white matter from the spinal cord piece were determined by the software. These volumes in the control and treated SCI groups were similar. Lesion volume was calculated by subtracting preserved tissue volumes from the total cord volume. Four control and five treated samples were used for these analyses.

Statistical analysis

Mean values are expressed as ± standard error (SE). Results were subjected to parametric statistical analysis using analysis of variance (ANOVA; Snedecor and Cochran, 1989). A one-way ANOVA was used to analyze locomotor scores and hindpaw withdrawal responses, as these measures reached a plateau with time, and changes with time and treatment did not interact. One-way ANOVA was also used for comparison of marker expression and activity associated with leukocyte infiltration and oxidative stress, as well as histopathological parameters. Differences between means were determined by Student-Neuman-Keuls (SNK) testing. Analysis of the histological tissue sparing was done by Student's *t*-tests. Significance was accepted at $p < 0.05$. p Values given in the results section for all but the histological data refer to the values obtained in the SNK test following the ANOVA. Details of the ANOVA are provided in the figure legends. All analyses were done with the investigators blinded to the treatment the rats had received.

Results

PDE4-I treatment reduces intraspinal neutrophils and macrophages after SCI

Rats were treated with PDE4-I at three different doses (0.5, 1.0, and 3.0 mg/kg), delivered in bolus doses at 2 and 12 h after SCI (effects were assessed at 24 h), or at 12-h intervals up

to 60 h after injury (effects were assessed at 72 h). The effect of this PDE4-I treatment on neutrophil influx and macrophage activation after SCI was quantified using an MPO activity assay. MPO enzymatic activity (6.90 ± 0.02 units/g tissue) was minimal in the uninjured spinal cord (Fig. 1A). At 24 and 72 h after SCI, MPO activity in the T2–T6 cord of the control SCI group increased significantly (by 11-fold at 24 h; by eightfold at 72 h) compared to the uninjured controls. Treatment with 0.5 mg/kg PDE4-I significantly reduced MPO activity (−44%, $p = 0.002$ and −38.3%, $p = 0.018$) at both 24 h and 72 h post-injury compared to the SCI controls. At 1.0 mg/kg, the PDE4-I significantly reduced MPO activity at both 24 h and 72 h post-injury (−29.8%, $p = 0.024$ and −39.1%, $p = 0.017$, respectively). After 3.0 mg/kg PDE4-I, MPO activity was reduced significantly only at 24 h post-injury (−23.8%, $p = 0.038$, Fig. 1A). Whereas the PDE4-I treatments reduced MPO activity, they did not return it to levels found in uninjured rats, as MPO activity in all groups of SCI rats remained greater than that in the uninjured rats. The treatment effects on MPO activity did not differ significantly by dose.

To complement the MPO assay, the presence of neutrophils in the cord was quantified using an anti-neutrophil antibody in a Western blot analysis. Neutrophil protein (molecular weight 56 kDa) expression was low in uninjured spinal cord homogenates (Fig. 1B), but increased significantly at 24 and 72 h after SCI. Treatment with 0.5 mg/kg PDE4-I significantly reduced neutrophil protein expression, by 52% ($p = 0.002$) at 24 h, and by 57% ($p = 0.038$) at 72 h after injury compared to SCI controls. The 1.0-mg/kg dose of PDE4-I significantly reduced neutrophil protein expression only at 24 h post-injury (−45%, $p = 0.016$). The 3.0-mg/kg dose did not reduce protein expression at either sampling time point. When effects of different PDE4-I doses on neutrophil infiltration were compared, the effects of the 0.5-mg/kg and 1.0-mg/kg doses were not different at 24 h or 72 h post-injury. However, at 72 h post-injury the 0.5-mg/kg dose tended to have a greater effect on the neutrophils than the 3.0-mg/kg dose ($p = 0.09$, Fig. 1B). No difference was found among the three doses at 24 h post-injury. Like the MPO assay, the neutrophil antigen expression

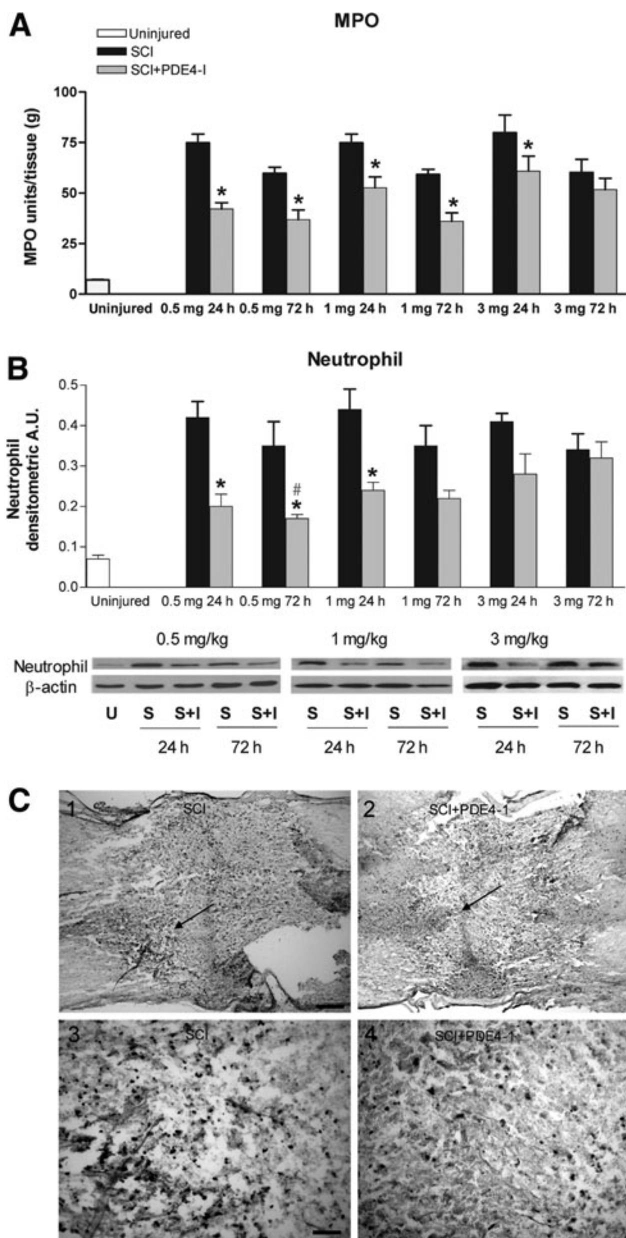


FIG. 1. Phosphodiesterase type 4 inhibitor (PDE4-I) treatment decreases intraspinal neutrophil and monocyte/macrophage influx at 24 h and 72 h after SCI. (**A**) Myeloperoxidase (MPO) activity in cord homogenates increased after injury in control rats ($n = 4/5$ per group to match treated rat numbers), and in the PDE4-I-treated rats compared to uninjured rats ($n = 7$). These increases were significantly smaller in the PDE4-I-treated rats than in controls at the doses of 0.5 mg/kg or 1.0 mg/kg at 24 h and 72 h post-injury ($n = 4$–5/group; see Table 1), and 3.0 mg/kg at 24 h ($n = 4$/group), but not 72 h ($n = 4$/group) after SCI (analysis of variance [ANOVA] $F_{12,50} = 19.08$, $p < 0.001$; Student-Neuman-Keuls [SNK] test, $p < 0.05$). Values are means ± standard error (SE; *significantly different from control injured). All groups were significantly different from uninjured (SCI, SCI control injured; SCI + PDE4-I, treated injured). (**B**) Neutrophil protein, as identified by Western blotting, in cord homogenates and expressed in arbitrary units (A.U.), increased after injury in control rats and in the PDE4-I-treated rats compared to uninjured rats ($n = 4$ for all groups). Representative autoradiograms of Western blots are shown in the bottom panel (U, uninjured; S, SCI, injured; S + I, injured + treated). These increases were significantly smaller in the PDE4-I-treated rats than in controls at the doses of 0.5 mg/kg or 1.0 mg/kg at 24 h post-injury, and 0.5 mg/kg at 72 h after SCI (ANOVA, $F_{12,39} = 8.5$, $p < 0.001$; by SNK test $p < 0.05$). The MPO activity at 72 h post-injury in the 0.5-mg/kg treatment group tended to be smaller that in the 3.0-mg/kg treatment group ($p = 0.09$). Values are means ± SE (*significantly different from control injured; #tended to differ from 3.0-mg/kg PDE4-I treated at 72 h). All groups were significantly different from uninjured animals. Abbreviations as above. (**C**) Photomicrographs of longitudinal sections of spinal cord immunostained by an anti-neutrophil antibody. The sections from control injured (panels **C1** and **C3**), and 0.5-mg/kg PDE4-I-treated rats (panels **C2** and **C4**) at 72 h following T4 clip compression injury. Panels **C3** and **C4** show detail of area indicated by the arrows in panels **C1** and **C2**, respectively (scale bar = 500 μm in **C1** and **C2** and 100 μm in **C3** and **C4**). The section from the PDE4-I-treated rat appears to have fewer neutrophils.

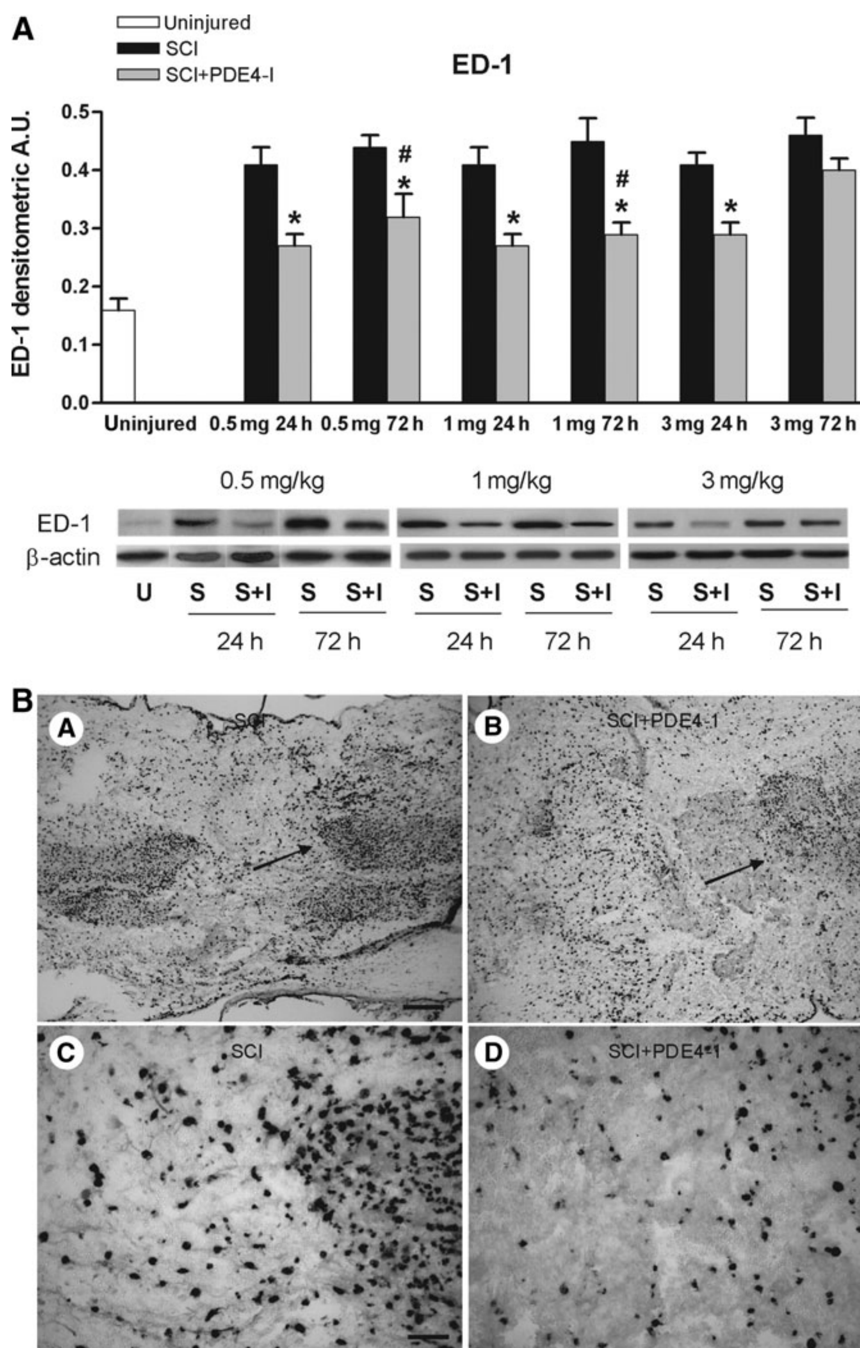


FIG. 2. Phosphodiesterase type 4 inhibitor (PDE4-I) treatment decreases intraspinal monocyte/macrophage influx at 24 h and 72 h after SCI. (**A**) Western blotting with ED-1, expressed in arbitrary units (A.U.), in cord homogenates ($n = 4$–6/group; see Table 1) showed an increase at 24 and 72 h after SCI compared to uninjured cords; these increases were reduced by PDE4-I administration (analysis of variance [ANOVA] $F_{12,39} = 11.26$, $p < 0.001$; Student-Neuman-Keuls [SNK] test $p < 0.05$). A representative autoradiogram of a Western blot is shown in the bottom panel Values are means ± standard error (SE; *significantly different from control injured; #significantly different from 3-mg/kg PDE4-I treated at 72 h; all groups were significantly different from uninjured animals; U, uninjured; S, SCI control injured; S + I, PDE4-I-treated injured; SCI + PDE4-I, treated injured). (**B**) Photomicrographs of longitudinal sections of spinal cord immunostained by an anti-ED1 antibody. The sections from control injured (**A** and **C**), and PDE4-I-treated rats (0.5 mg/kg, **B** and **D**) at 72 h following T4 clip-compression injury. **C** and **D** show detail of area indicated by the arrows in **A** and **B**, respectively (scale bars = 500 μm in **A** and **B**, and 100 μm in **C** and **D**). The section from the PDE4-I-treated rat appears to have fewer monocytes/macrophages.

in the injured spinal cords remained greater than in uninjured rats after all PDE4-I treatments.

To corroborate the Western blot results the anti-neutrophil antibody was used to identify neutrophils in the injured spinal cord. At 72 h after SCI, many cells that were stained by the anti-neutrophil antibody were detected in the lesion site of the spinal cord (Fig. 1 panels C1 and C3), and after PDE4-I treatment (0.5 mg/kg) the density of these cells appeared to be reduced (Fig. 1 panels C2 and C4).

The presence of phagocytic macrophages in the injured spinal cord was estimated using Western blot analysis for ED-1 expression (molecular weight 110 kDa) in cord homogenates. ED-1 expression was low in uninjured spinal cord (Fig. 2A), but increased significantly at 24 and 72 h after SCI. Treatment with 0.5 mg/kg PDE4-I significantly reduced ED-1 expression (−34%, $p=0.014$ and −27%, $p=0.039$) at both 24 h and 72 h after injury compared with SCI controls. A dose of 1.0 mg/kg of the PDE4-I also significantly reduced ED-1 expression at both 24 and 72 h post-injury (−34%, $p=0.015$ and −36%, $p=0.004$, respectively). Like MPO activity, 3.0 mg/kg PDE4-I only reduced ED-1 expression at 24 h post-injury (−29%, $p=0.021$). When the effects of different PDE4-I doses on macrophage infiltration were compared, the 0.5-mg/kg and 1.0-mg/kg doses were not different at 24 h or 72 h post-injury. However, significant differences were found between the 0.5-mg/kg and 3.0-mg/kg doses ($p=0.05$) at 72 h post-injury, and between the 1.0-mg/kg and 3.0-mg/kg doses ($p=0.029$) at 72 h post-injury (Fig. 2A). No difference was found among the three doses at 24 h post-injury. To corroborate the Western blot results, an anti-ED-1 antibody was used to identify macrophages in the injured spinal cord. At 72 h after SCI, many ED-1-immunoreactive (IR) cells were detected in the lesion site of the spinal cord (Fig. 2B), and after PDE4-I treatment (0.5 mg/kg) the density of these cells clearly appeared to be reduced (Fig. 2B).

Lipid peroxidation after SCI decreases with PDE4-I treatment

Next, effects of these doses of PDE4-I on spinal cord lipid membrane damage were evaluated by examining lipid peroxidation (malondialdehyde) in homogenates of the injured

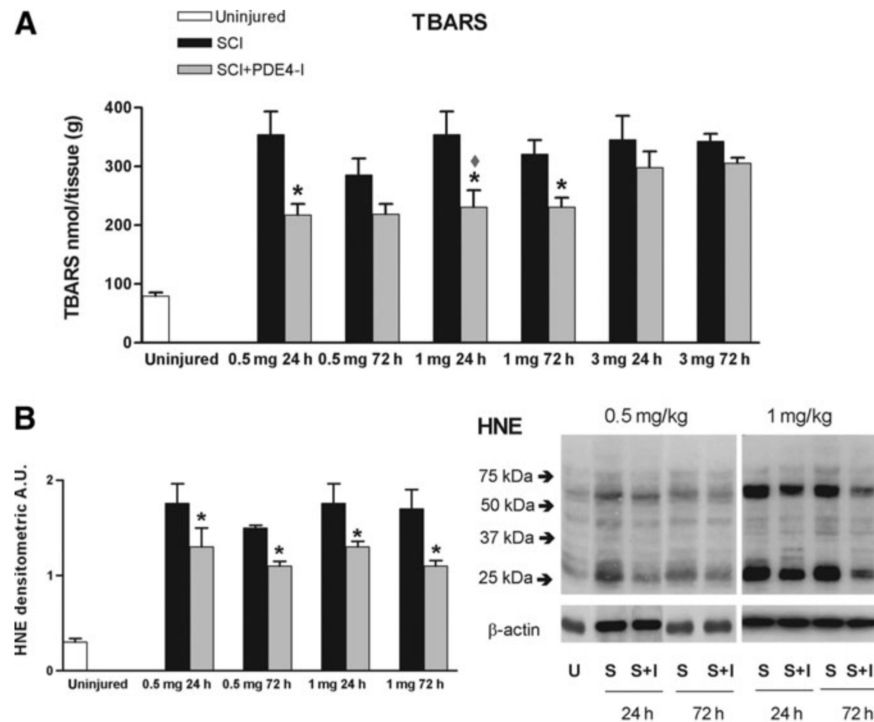


FIG. 3. Phosphodiesterase type 4 inhibitor (PDE4-I) treatment decreased lipid peroxidation in spinal cord homogenates at 24 and 72 h after spinal cord injury (SCI). Lipid peroxidation was assessed using the thiobarbituric acid reactive substances (TBARS) assay for malondialdehyde. **(A)** TBARS concentration increased significantly in controls ($n=4$–5 to match numbers in the treated groups), and the PDE4-I-treated rats ($n=4$–5; see Table 1), when compared to that in the cords of uninjured rats ($n=6$). In rats treated with PDE4-I at the 0.5-mg/kg and 1.0-mg/kg doses, the increases were significantly smaller than those of controls at 24 and 72 h after injury (analysis of variance [ANOVA] $F_{12,49}=13.69$, $p<0.001$; Student-Neuman-Keuls [SNK] test $p<0.05$). After the 3.0-mg/kg dose ($n=4$) the TBARS concentration was not significantly changed. The TBARS concentration at 24 h after injury in the 1.0-mg/kg treatment group tended to be smaller than that in the 3.0-mg/kg group ($p=0.08$). Lipid peroxidation was also assayed by Western blotting for 4-hydroxynonenol (HNE, **B**, $n=4$–6/group; see Table 1), and the PDE4-I treatment reduced HNE protein damage significantly (ANOVA $F_{8,27}=44.56$, $p<0.001$; SNK test $p<0.05$). Western blots illustrate HNE-bound protein expression compared to loading controls (β-actin). Values are means ± standard error (SE). All groups were significantly different from uninjured animals (*significantly different from SCI controls; ♦ tended to differ from 3-mg/kg PDE4-I-treated at 24 h; U, uninjured; S, SCI control injured; S + I, PDE4-I-treated injured; SCI + PDE4-I, treated injured).

cord. Lipid peroxidation, as assessed by the amount of TBARS in the cord homogenate, was minimal in the uninjured spinal cord (79 ± 6 nmol TBARS/g tissue; Fig. 3A). The TBARS concentration was significantly increased (by 3.6–4.4-fold) in SCI controls at 24 and 72 h after injury (285–350 nmol/g tissue). Treatment with 0.5 mg/kg PDE4-I significantly reduced TBARS levels at 24 h after SCI (−38%, $p = 0.012$), but not at 72 h. Administration of 1.0 mg/kg PDE4-I significantly reduced TBARS levels (−35%, $p = 0.005$ and −28%, $p = 0.059$) at 24 and 72 h, respectively, after SCI. The 3.0-mg/kg dose did not significantly reduce lipid peroxidation at 24 h or 72 h after SCI (Fig. 3A). When examining the dose response for this treatment using the TBARS concentration as an end-point, we found no significant difference between doses at 24 h or 72 h post-injury. The effects of 1.0 mg/kg tended to be greater than those of 3.0 mg/kg at 24 h post-injury ($p = 0.08$, Fig. 3A). As the highest dose of the PDE4-I (3 mg/kg) had less consistent effects on inflammation and intraspinal damage after SCI than the lower doses, we examined the 0.5- and 1.0-mg/kg doses on additional measures of lipid peroxidation, oxidative activity, functional outcomes, and histological damage of the injured cord.

The effects of 0.5 and 1.0 mg/kg PDE4-I on lipid peroxidation were also measured by the analysis of HNE-modified proteins using Western blotting. Very little HNE-modified protein was observed in the uninjured spinal cord (Fig. 3B). SCI, however, led to the modification of proteins both at 24 and 72 h after injury. Treatment with 0.5 mg/kg PDE4-I significantly decreased SCI-induced HNE protein modification (−26%, $p < 0.001$ and −17%, $p = 0.002$, respectively) at both 24 h and 72 h post-injury. The PDE4-I at 1.0 mg/kg reduced HNE protein modification at both 24 and 72 h after SCI (−29%, $p < 0.001$ and −38%, $p < 0.001$, respectively; Fig. 3B).

Free radical formation after SCI decreases with PDE4-I treatment

We used DCFH-DA as a probe for free radical generation and observed low concentrations of the oxidized DCF product in uninjured cord homogenates (Fig. 4). In contrast, DCF production in injured controls was significantly increased, by up to fivefold ($p < 0.001$), at both 24 and 72 h after SCI. Treatment with 0.5 mg/kg PDE4-I significantly lowered DCF concentration in the injured cord at both 24 h and 72 h after injury (−29% and −26%, $p = 0.032$ and $p = 0.014$, respectively). The PDE4-I at 1.0 mg/kg significantly reduced DCF levels at 24 and 72 h (−34%, $p = 0.007$ and −26%, $p = 0.02$, respectively; Fig. 4).

Production of oxidative enzymes after SCI decreases with PDE4-I treatment

Expression of the oxidative enzymes gp91^{phox}, iNOS, and COX-2, was detected by Western blotting. Immunoblot detection of gp91^{phox} revealed a single band at 58 kDa in all groups, with low expression in uninjured cords (Fig. 5A). At 24 and 72 h after SCI, expression of gp91^{phox} significantly increased by fourfold compared to uninjured controls. The administration of 0.5 mg/kg PDE4-I significantly reduced gp91^{phox} (−24% $p = 0.026$ and −21% $p = 0.05$, respectively) at 24 h and 72 h after SCI compared to SCI controls. Delivery of 1.0 mg/kg PDE4-I reduced gp91^{phox} at both 24 and 72 h post-injury (−21%, $p = 0.014$ and −35%, $p < 0.001$, respectively; Fig. 5A).

Western blotting for iNOS revealed a band at 130 kDa that was faint in uninjured cords (Fig. 5B). A significant increase in iNOS protein expression (sevenfold) was observed at 24 and 72 h after SCI. Treatment with 0.5 mg/kg PDE4-I significantly decreased iNOS expression (−29%, $p < 0.001$ and −39%, $p < 0.001$, respectively) at both 24 h and 72 h post-injury. The administration of 1.0 mg/kg PDE4-I significantly reduced iNOS expression at both 24 and 72 h post-injury (−46%, $p < 0.001$ and −40%, $p < 0.001$, respectively), compared to SCI controls (Fig. 5B).

COX-2 was identified at a molecular weight of 72 kDa, again with very low expression in uninjured controls (Fig. 5C). At 72 h after SCI, COX-2 expression significantly increased, by eightfold, and treatment with 0.5 mg/kg and 1.0 mg/kg PDE4-I significantly reduced COX-2 (−25%, $p = 0.016$ and −15%, $p = 0.041$, respectively) compared to the SCI controls (Fig. 5C).

PDE4-I treatment improves motor function after SCI

The 0.5- and 1.0-mg/kg doses of PDE4-I that had demonstrated the most robust anti-inflammatory and antioxidant effects were used to evaluate effects on locomotor performance using the BBB scoring method (Basso et al., 1995). The 0.5-mg/kg dose of PDE4-I produced a significant improvement in BBB scores that persisted from 4–8 weeks post-injury (Fig. 6A; $p = 0.03$–0.05). The scores differed by an average of 1.3 points during this time. A significant effect of 1.0 mg/kg PDE4-I treatment was detected by ANOVA ($p < 0.001$), but the mean values of the control and treatment groups did not differ significantly. The difference between the values for control and treated rats at the 1.0-mg/kg dose during the 4- to 8-week interval averaged only 0.6 point. By interpreting these scores, we saw that the control rats in our study were able to

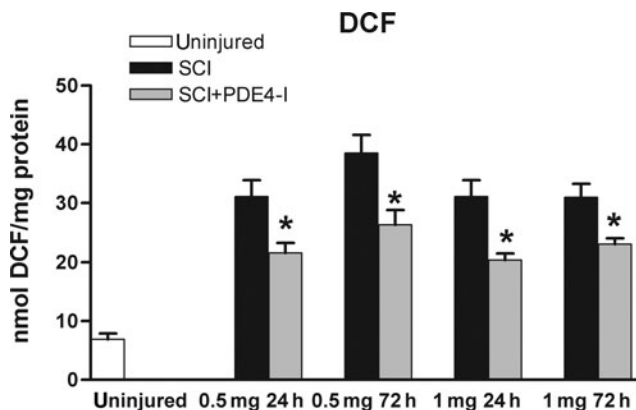
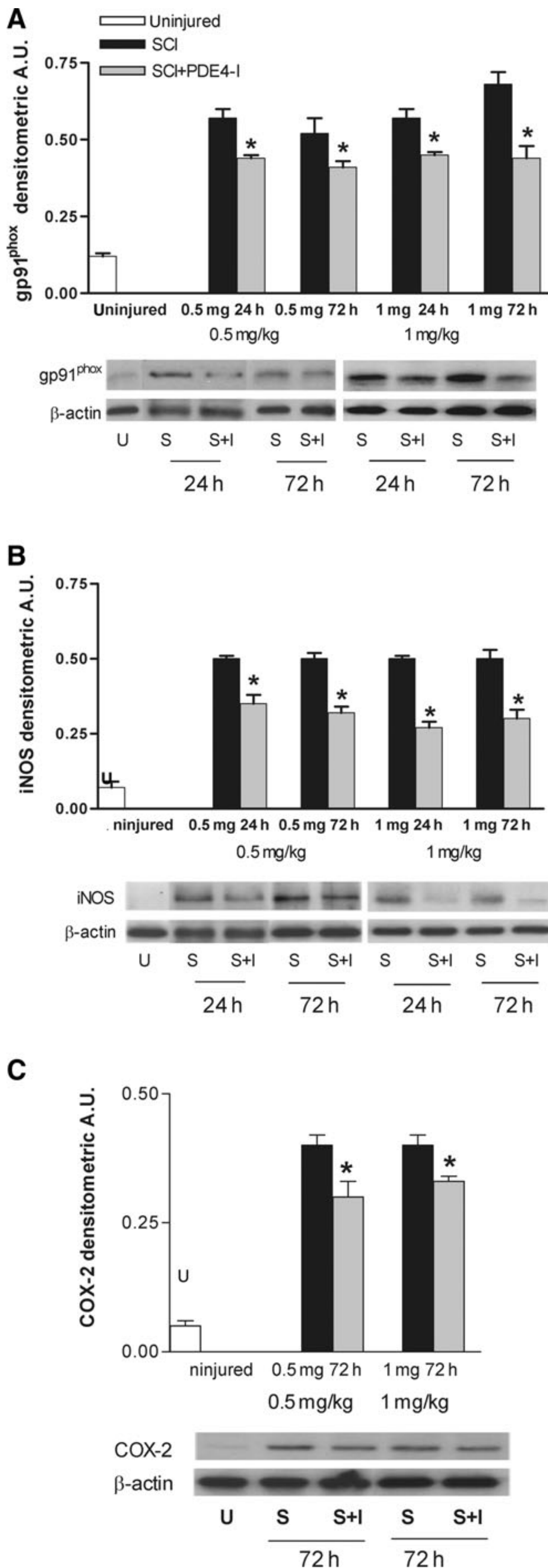


FIG. 4. Phosphodiesterase type 4 inhibitor (PDE4-I) treatment decreased 2′-7′-dichlorofluorescein (DCF) fluorescence, a marker of reactive oxygen species, in cord homogenates at 24 and 72 h after spinal cord injury (SCI). DCF levels increased in the injured controls ($n = 5$–6/group; see Table 1), and in the PDE4-I-treated rats ($n = 5$/group) compared to uninjured rats ($n = 6$). DCF levels, however, were significantly lower in the PDE4-I-treated rats than in controls, both at 0.5-mg/kg and 1.0-mg/kg doses (analysis of variance [ANOVA] $F_{8,35} = 21.18$, $p < 0.001$; Student-Neuman-Keuls [SNK] test $p < 0.05$). Values are means ± standard error (*significantly different from controls). All groups were significantly different compared to uninjured animals.



move their hindlimbs extensively, often in a sweeping movement, but rarely achieved a weight-supporting stance. The 0.5-mg/kg PDE-4-I-treated rats were able to support their weight and to make dorsal or plantar-stepping movements that permitted locomotion without coordination of forelimb and hindlimb movements.

PDE4-I treatment decreases mechanical allodynia after SCI

Mechanical allodynia is a form of neuropathic pain that develops after neural injury in which an innocuous stimulus is perceived as painful; in animals allodynia can be identified by their avoidance responses to touch (Christensen et al., 1996). Mechanical allodynia was assessed on the hindpaws both before, and at 2–6 weeks after, SCI using a 15-mN modified Semmes-Weinstein filament (Fig. 6B). Before SCI, the rats did not exhibit avoidance responses to stimulation by the filament. Following SCI, control animals showed numbers of avoidance responses to paw stimuli (maximum 3.3 ± 0.3), consistent with the development of mechanical allodynia (Oatway et al., 2005). Administration of PDE4-I significantly decreased the frequency of the avoidance responses to hindpaw stimulation (0.5 mg/kg, $p < 0.001$, maximum 2.6 ± 0.6; 1.0 mg/kg, $p < 0.001$, maximum 2.3 ± 0.4) from 4–6 weeks after injury (Fig. 6B). At 2 weeks after SCI, the 1.0-mg/kg dose also reduced the number of avoidance responses ($p = 0.05$), and the 0.5-mg/kg dose also tended to reduce them ($p = 0.07$). The mechanical allodynia scores of rats receiving the 0.5-mg/kg dose of PDE4-I were similar to the scores of those receiving the higher dose.

Preservation of tissue by PDE4-I treatment

We assessed tissue sparing in the rats that had undergone 8 weeks of behavioral testing, examining the group that had the best motor improvement (dosed at 0.5 mg/kg). Sparing of neurons and the volume of gray matter, white matter, and lesion size within the T2–T6 spinal cord were examined after PDE4-I treatment and compared to SCI controls using

FIG. 5. Phosphodiesterase type 4 inhibitor (PDE4-I) treatment decreases the production of oxidative enzymes in cord homogenates at 24 and 72 h after spinal cord injury (SCI). When assayed by Western blotting, expression of (**A**) gp91^{phox}, a subunit of reduced nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase ($n = 4$–6/group; see Table 1), (**B**) inducible nitric oxide synthase (iNOS; $n = 4$–6/group), and (**C**) cyclooxygenase-2 (COX-2; $n = 4$–6/group) increased after SCI (both controls and PDE4-I-treated animals) compared to uninjured rats. See Table 1 for details of animal numbers. These increases were significantly smaller in the PDE4-I-treated rats than in controls with the 0.5-mg/kg and 1.0-mg/kg doses at 24 and 72 h after SCI (analysis of variance [ANOVA]: gp91^{phox} $F_{8,27} = 30.1$, $p < 0.001$; iNOS $F_{8,27} = 62.2$, $p < 0.001$; COX-2 $F_{4,15} = 57.7$, $p < 0.001$). All comparisons of means by Student-Neuman-Keuls test ($p < 0.05$). Representative Western blots illustrate gp91^{phox}, iNOS, and COX-2 expression compared to loading controls (β-actin). Values are means ± standard error (all groups were significantly different from uninjured animals; *significantly different from controls; U, uninjured; S, SCI control injured; S + I, PDE4-I-treated injured; SCI + PDE4-I, treated injured).

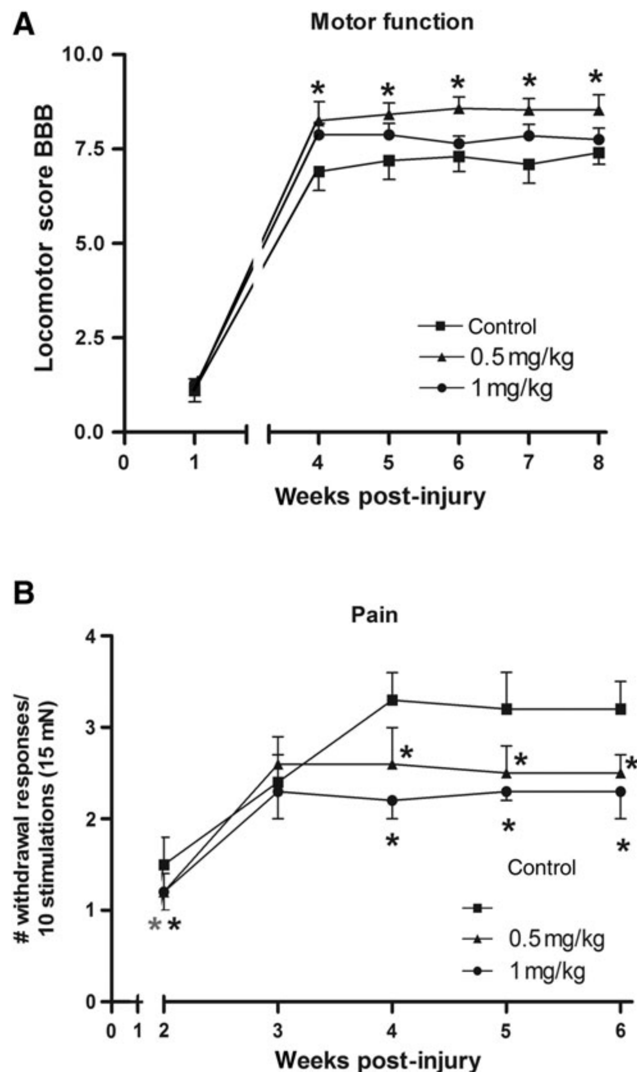


FIG. 6. Phosphodiesterase type 4 inhibitor (PDE4-I) treatment improves locomotor function and decreases mechanical allodynia after spinal cord injury (SCI). Basso-Beattie-Bresnahan (BBB) locomotor scores (**A**) were obtained from the PDE4-I-treated injured rats and SCI controls twice weekly for 8 weeks. The left and right leg scores were averaged after each test, and the two scores per week were averaged. Rats receiving the 0.5-mg/kg dose ($n=6$) had BBB scores significantly greater than those of SCI controls ($n=11$; see Table 1) from 4–8 weeks after SCI (analysis of variance [ANOVA] $F_{11,90}=73.5$, $p<0.001$; Student-Neuman-Keuls [SNK] test $p<0.05$). BBB scores for the PDE4-I-treated rats at 1.0 mg/kg ($n=6$) were not different from those of SCI controls (ANOVA $F_{11,90}=78.0$, $p<0.001$; SNK test $p<0.05$). (**B**) Mechanical allodynia was detected by avoidance responses to hindpaw stimulation in SCI control rats ($n=11$), and in PDE4-I-treated injured rats ($n=6$). The rats were tested once per week, commencing at 2 weeks after SCI; scores of the left and right hindpaws were averaged. The PDE4-I treatment caused significant decreases in avoidance responses at 1 week (1.0-mg/kg group only) after SCI, and at 4–6 weeks (both doses) after SCI (0.5 mg/kg ANOVA $F_{9,75}=45.3$, $p<0.001$; SNK test $p<0.05$; 1.0 mg/kg ANOVA $F_{9,75}=54.0$, $p<0.001$; SNK test $p<0.05$). Values are means ± standard error (*significantly different between control and PDE4-I-treated rats; *0.05 mg/kg PDE4-I-treated rats tended to differ from controls, $p=0.07$).

stereological quantification of cells immunoreactive for the neuron marker NeuN and H&E-stained tissue sections at 8 weeks post-injury (Fig. 7). In the rats receiving PDE4-I treatment, overall tissue integrity at the lesion site was more intact than in the control spinal cords (Fig. 7 top). Apparent sparing of both gray and white matter was visible upon microscopic inspection of the tissue sections. Indeed, PDE4-I treatment significantly reduced the loss of white matter, as the volume of white matter was greater than that in the controls by 38% ($p=0.004$ by Student's *t*-test). Although the treatment decreased the lesion volume by 21%, this change was not significant ($p=0.27$ by Student's *t*-test). In contrast, the number of preserved NeuN-immunoreactive cells did not change when compared with the numbers in controls ($p=0.24$ by Student's *t*-test; Fig. 7 bottom). Likewise the volume of gray matter at the lesion site did not change significantly ($p=0.8$ by Student's *t*-test).

Discussion

When given within the first 72 h after SCI, the PDE4-I (IC486051) effectively decreased inflammatory cell infiltration and activity in the injured spinal cord, reduced intraspinal free radical formation and oxidative damage, preserved white matter, and improved neurological function after SCI. Although the previous therapeutic use of PDE4-I inhibitors after SCI has largely focused on their support of axonal growth, particularly in combination with cellular implants (Bretzner et al., 2010; Nikulina et al., 2004; Pearse et al., 2004a), the present study shows that when acutely administered they can also provide neuroprotection, likely through an anti-inflammatory action. This finding correlates well with our previous observations that brief, early anti-inflammatory treatments can reduce both immune cell infiltration and oxidative damage within the injured cord, leading to improved motor and autonomic outcomes, as well as reduced neuropathic pain after SCI (Bao et al., 2004a, 2004b; Gris et al., 2004). It also is consistent with studies of others who noted neuroprotective effects after long-lasting treatments with rolipram (Beaumont et al., 2009; Iannotti et al., 2010; Pearse et al., 2004a; Whitaker et al., 2008).

All markers of inflammation and its secondary consequences examined in our study were decreased by the early administration of the PDE4 inhibitor IC486051. Although we did not count infiltrating neutrophils and monocyte/macrophage microglial activation, we employed Western blotting for their proteins and enzymatic markers known to be present almost exclusively on specific immune cell types within the injured CNS. MPO, an oxidative enzyme largely produced by neutrophils, ED-1, a lysosomal protein expressed by activated macrophages and microglia (Bao et al., 2004b), and the neutrophil-associated protein were substantially reduced by PDE4-I at 24 h and 72 h post-injury. The reduction of MPO activity by 72 h post-injury may also be attributed in part to effects on the influx of monocytes/macrophages into the injured cord. The first 72 h after injury coincides with the entry and activation of these inflammatory leukocytes (Saville et al., 2004). Immune cell activation after SCI leads to a well-described upregulation of enzymes associated with oxidative damage, including $gp91^{phox}$, iNOS, and COX-2 (Bao et al., 2004a, 2004b, 2005). Administration of the PDE4-I reduced these responses substantially, as well as the ensuing production

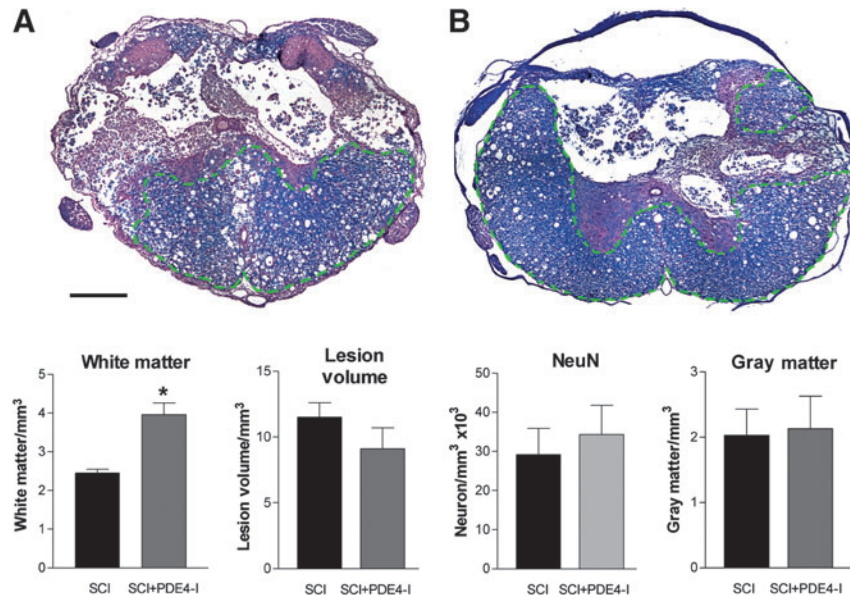


FIG. 7. Phosphodiesterase type 4 inhibitor (PDE4-I) treatment at 0.5 mg/kg preserves tissue at 8 weeks after SCI. The top panel shows photomicrographs of Luxol fast blue- and hematoxylin and eosin (H&E)-stained cross-sections of spinal cord from control (**A**) and PDE4-I-treated rats (**B**). Normal-appearing white matter used for quantification is outlined with a green line. Note the greater overall tissue preservation in the spinal cord of the PDE4-I-treated rat (scale bar = 500 μm). The volumes of gray matter, white matter, and lesion size and neuron number within the T3–T5 spinal cord of controls ($n = 5$) and PDE4-I-treated rats ($n = 5$) are plotted in the lower panel. PDE4-I treatment significantly increased the white matter volume ($p = 0.004$ by Student's *t*-test), but did not significantly change gray matter, lesion volume, or neuron number. The coefficient of error for the counts of neurons ranged from 0.024–0.048 among the control and PDE4-I-treated rats, and the average value was 0.032 (SCI, SCI control injured; SCI + PDE4-I, treated injured).

of free radicals. Importantly, reductions in inflammatory and oxidative processes by PDE4-I were accompanied by a significant abrogation of lipid peroxidation at 24 and 72 h after SCI, as well as white-matter preservation at 8 weeks. Neuroprotective effects reported after treatment with rolipram include sparing of oligodendrocytes, increased central myelination (consistent with our white-matter sparing), and improved neurotransmission through the ventrolateral funiculus (Beaumont et al., 2009; Pearse et al., 2004a; Whitaker et al., 2008). These could be downstream benefits of the reduction of inflammation and oxidative injury caused by the PDE4 inhibitor. Prior to our investigation with IC486051, we conducted a small pilot study using rolipram and found that it limited intraspinal inflammation and oxidative injury after SCI (Weaver and Bao, unpublished observations). The anti-inflammatory and antioxidant effects of PDE4 inhibition in our study may also have translated into oligodendrocyte preservation and improved neurotransmission, as possible downstream mechanisms for the functional improvements that we observed.

The early protective actions of the PDE4-I led to long-term improvements in locomotor function and a reduction in neuropathic pain, similarly to effects we have observed with other neuroprotective treatments (Fleming et al., 2008; Gris et al., 2004). Previously, another PDE4 inhibitor, rolipram, when administered subcutaneously for 2 weeks, improved BBB scores by about 2 points (from approximately 10–12) at 5 weeks after injury when delivery was initiated within 15 min of a moderate T8 thoracic SCI (Pearse et al., 2004a). In the more severe injury model employed in the current study, the 1-point improvement in BBB scores observed with the PDE4-I following a 72-h treatment protocol compares favorably with the outcome of the protracted treatment after a milder injury.

We demonstrated no consistent statistically significant differences between the three doses of the PDE4-I tested. But, in contrast the 0.5- and 1.0-mg/kg doses, the 3.0-mg/kg dose had a smaller anti-inflammatory effect at 24 h, and no anti-inflammatory effect by 72 h after SCI. We opted to test only the doses with more robust actions in later histological and behavioral investigations. A lack of persistent anti-inflammatory action with dose escalation may be due to the putative activation of opposing intracellular signaling pathways associated with the many targets of cAMP (Borland et al., 2009). Moreover, increased cAMP and inhibited PDE4 activity have been shown to have proinflammatory actions in certain circumstances (Hertz et al., 2009), and perhaps at high doses, this kind of action confounded any positive effects. Although both 0.5 and 1.0 mg/kg of the PDE4-I ameliorated mechanical allodynia (neuropathic pain) for the duration of the study, the lowest dose had the most robust effect on locomotor function. These outcomes correlated with significant sparing of white matter at 8 weeks after the injury. The lack of a significant reduction in lesion volume was consistent with the lack of grey matter and neuronal sparing. A conclusion from these findings may be that, rather than rescue of neurons and glia, sparing of axons across the lesion or promotion of their sprouting is a more important factor in the neuroprotection afforded by this treatment. Labeling studies to examine the integrity of descending axon systems would help to answer this question. Moreover, the significant white-matter sparing that we observed is consistent with the protection of oligodendrocytes, and greater central myelination and neurotransmission, outcomes reported

by others after treatment with the PDE4 inhibitor rolipram (Beaumont et al., 2009; Pearse et al., 2004a; Whitaker et al., 2008). Another mechanism by which the acute PDE4-I treatment could promote regeneration of these axons would be by protecting against secondary damage and scarring within the lesion site due to reduction of oxidative activity. In our study, direct treatment effects would no longer be present during the critical periods for regeneration.

Increased cAMP concentrations have been implicated in the inhibition of proinflammatory cell functions such as chemotaxis, degranulation, superoxide anion generation, release of IL-8, and phagocytosis in neutrophils (Ottonello et al., 1995; Pearse et al., 2004a; Pryzwansky and Madden, 2003; Rossi et al., 1998). Furthermore, monocyte adhesion and migration are inhibited by high cAMP levels. Phagocytosis and nitric oxide production in macrophages are also downregulated (Aronoff et al., 2005; Rossi et al., 1998; Zhu et al., 2001). In addition, an increase in cAMP decreases the expression of adhesion molecules (CD11b/CD18/L-selectin), and adhesion to other cells, and disrupts chemokine-induced chemotaxis (Derian et al., 1995; Harvath et al., 1991). cAMP is thus a key intracellular second messenger that at increased levels can have anti-inflammatory and tissue-protective effects. cAMP levels are increased upon activation of the membrane G-protein coupled receptor subunit Gsα, that in turn activates adenylyl cyclase (Wiemelt et al., 1997). Adenylyl cyclase has a host of downstream effector systems important in cell signaling (Beavo and Brunton, 2002). cAMP is highly regulated in neutrophils and monocytes/macrophages through PDE-mediated cAMP degradation. Three of the four genes belonging to the PDE4 family (namely, PDE4A, PDE4B, and PDE4D) are ubiquitously expressed in inflammatory cells (Ariga et al., 2004). The PDE4 inhibitor treatment used in this study, IC486051, would prevent PDE4 from hydrolyzing cAMP, thus maintaining intracellular cAMP levels, an important stabilizing force when they are significantly reduced after CNS injury (Atkins et al., 2007; Pearse et al., 2004b). Unfortunately, we did not measure cAMP directly in the leukocytes of our rats, and must infer that the inhibition of PDE4 did stabilize or increase cAMP in these and other cells.

Increased cAMP signaling contributes to the inhibition of inflammatory responses by several possible mechanisms. Some evidence suggests that cAMP signaling is regulated by a protein kinase A (PKA)-dependent pathway. PDE4A, PDE4B, and PDE4D have been targeted and co-localized with the catalytic subunit of PKA in the forming phagosome (Pryzwansky and Madden, 2003). PKA phosphorylates many downstream targets that lead to inhibition of cell activation. For example, the cAMP-PKA pathway negatively regulates Rac activity, leading to reduced neutrophil migration (Nagasawa et al., 2005). PKA contributes to the suppression of TNF-α production (Pearse et al., 2004a; Zhu et al., 2001), and inhibition of apoptosis through the MAPK-ERK pathway (Martin et al., 2001). PKA also partially mediates inhibition of the NADPH oxidase assembly through a RAP1a-mediated pathway (Ottonello et al., 1995). cAMP may also foster inhibitory cellular effects by directly activating phospholipase D, arachidonic acid, or Ca^{2+} influx (Agwu et al., 1991; Pryzwansky and Madden, 2003). Aronoff and associates (Aronoff et al., 2005) showed that direct cAMP activation of the guanine exchange protein Epac-1 contributes to inhibition of macrophage phagocytic function, without the involvement of PKA.

The anti-inflammatory potential of other PDE4-specific inhibitors such as rolipram has been evaluated in other disease models. Rolipram delays the onset and severity of experimental autoimmune encephalopathy (EAE), an animal model of multiple sclerosis, by delaying the peak in levels of TNF-α and IL-6 produced by Th-1 lymphoctes (Dinter et al., 2000; Zhu et al., 2001). In addition, rolipram passes the blood–brain barrier readily and reduces its permeability in the spinal cord of mice with EAE, causing reduced tissue edema and inflammatory cell infiltration (Zhu et al., 2001). Furthermore, superoxide production and elastase release by neutrophils are inhibited by rolipram through cAMP-dependent re-sequestration of cytosolic Ca^{2+} (Anderson et al., 1998). In addition, rolipram inhibits the expression of the adhesion molecules CD11b and L-selectin on platelet activating factor-stimulated neutrophils and eosinophils *in vitro* (Berends et al., 1997). As described above, sustained treatment with rolipram improves the outcomes of SCI (Beaumont et al., 2009; Iannotti et al., 2010; Pearse et al., 2004a), but clinical studies have revealed that it has major negative side effects in humans (Bruno et al., 2004; Zhu et al., 2001). RP-73401, Ariflo, and CDP-840 are newly-developed selective PDE4 inhibitors with higher potency and lower toxicity that may have potential for treating SCI (Bruno et al., 2004).

In conclusion, these findings emphasize the value of PDE4 inhibition in the treatment of SCI. Not only does it promote cellular sparing, myelination, regeneration, and functional recovery when given for long periods as described previously (Beaumont et al., 2009; Bretzner et al., 2010; Iannotti et al., 2010; Nikulina et al., 2004; Pearse et al., 2004a; Whitaker et al., 2008), but early, brief treatment with the PDE4 inhibitor IC486051 proved to be a useful anti-inflammatory strategy that yielded substantial neuroprotection and improved neurological outcomes. An appealing treatment paradigm could be an acute, brief treatment with doses of a PDE4 inhibitor to provide early neuroprotection and sparing of damaged tissue, followed by a more sustained, lower-dose treatment to supply further neuroprotection and facilitate regeneration.

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Author Disclosure Statement

No competing financial interests exist.

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